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TITLE: *sarA* as a target for the treatment and prevention of staphylococcal biofilm-associated infection

PRINCIPAL INVESTIGATOR: Mark S. Smeltzer

CONTRACTING ORGANIZATION: University of Arkansas for Medical Sciences
Little Rock, AR 72205

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14. ABSTRACT <p>Genetic studies in the PI's laboratory have demonstrated that mutation of the staphylococcal accessory regulator (<i>sarA</i>) limits biofilm formation in <i>Staphylococcus aureus</i> to a degree that can be correlated with increased antibiotic susceptibility and an improved therapeutic outcome in biofilm-associated infections. The goal of this project is to take therapeutic advantage of this observation by identifying small molecule inhibitors of <i>sarA</i> expression and/or function that could be used together with conventional antibiotics to achieve the desired therapeutic outcome. This will require two sets of experiments, the first being to carry out a large scale screen of potential inhibitors to identify those that offer the most promise. This is being done using genetic reporter constructs proven to accurately reflect the functional status of <i>sarA</i>. The second is to then evaluate the therapeutic efficacy of the most promising inhibitors using established animal models of biofilm-associated infection. In the previous progress report, we identified 31 compounds of potential interest. Secondary screens of these compounds, including those focusing directly on biofilm formation, led us to focus on a single compound (ST014221). While we are continuing to the primary screen, we have continued to screen other sources including 24 additional compounds from TimTec that are structurally related to ST014221. This led to identification of a single compound that exhibits even greater activity than ST014221 in our reporter assay. However, we have been unable to carry out the secondary biofilm screen owing to solubility problems with this compound in our test medium. We are currently reaching out to medicinal chemistry collaborators to address this issue, thereby allowing us to prioritize these compounds with respect to each other. As additional compounds are screened, these will then be examined by direct comparison to the most promising of these compounds. This will put us in a position to undertake the second objective of evaluating the impact of the best inhibitors with respect to both inhibiting <i>S. aureus</i> biofilm formation and relative antibiotic susceptibility in the specific context of an established biofilm.</p>					
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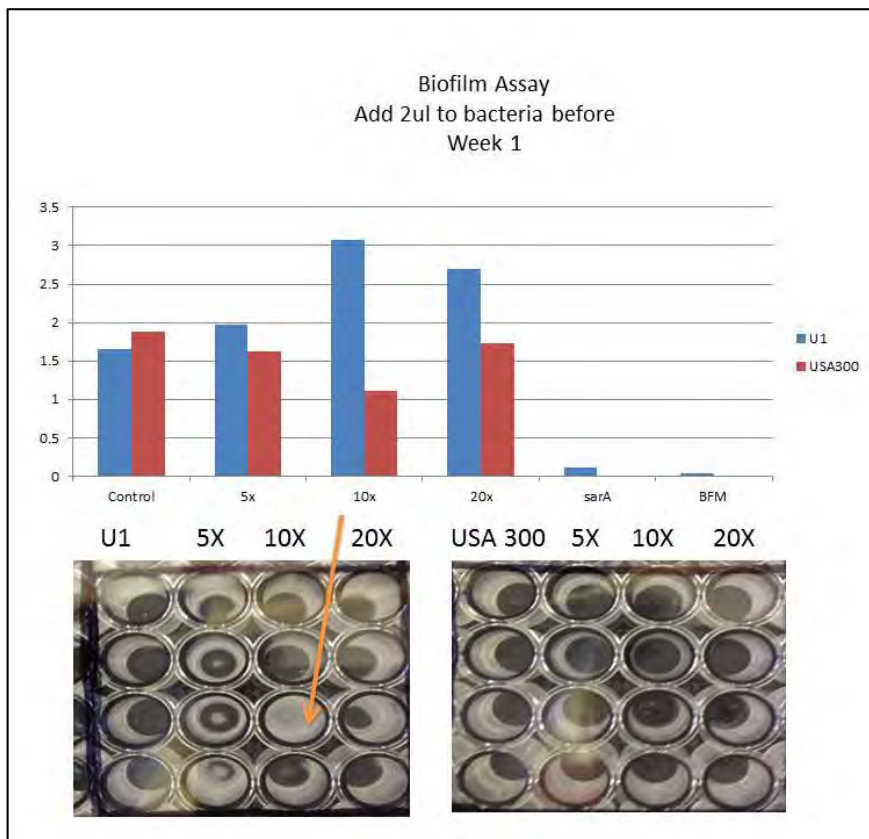
PROGRESS REPORT

Our research efforts in the last year have continued to focus on identifying small molecule inhibitors of the expression and/or function of the staphylococcal accessory regulator (*sarA*). The specific hypothesis is that such inhibitors could be used together with conventional antibiotic therapy to overcome the current limitations in the treatment of bone and implant-associated infections caused by *Staphylococcus aureus*. This focus on *S. aureus* is based on the fact that it is the leading cause of the most clinically problematic bone and implant-associated infections including those resulting from traumatic injuries incurred on the battlefield. The focus on *sarA* is based on the observations that 1) these infections are remarkably difficult to treat with conventional antibiotic therapy alone owing to formation of a biofilm on the affected tissues and/or implanted orthopaedic devices required to repair the injury and restore structural stability, and 2) mutation of *sarA* has been shown to limit biofilm formation to a degree that can be correlated with increased antibiotic susceptibility and an improved therapeutic outcome (1-3, 8, 9).

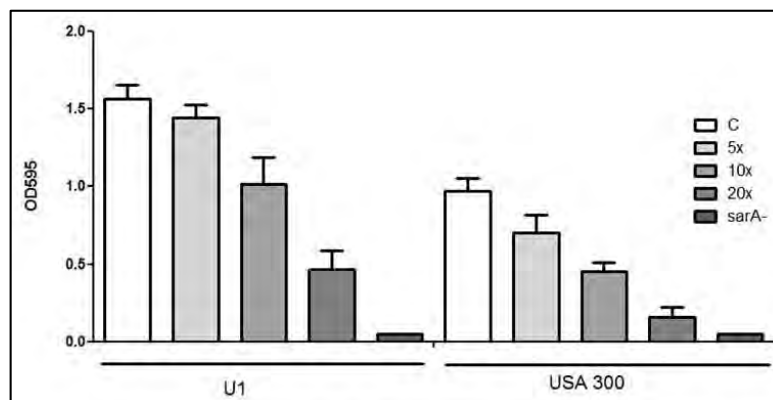
The overall objectives of the project are to first identify inhibitors and then evaluate their therapeutic promise using established animal models of biofilm-associated infection. As in the previous progress report, our efforts have continued to focus on screening a small molecule library that was previously shown to contain promising antimicrobial agents (7) to identify inhibitors of *sarA*-mediated biofilm formation. This library contains ~30,000 compounds, and it is not technically feasible to screen this many compounds based on biofilm formation itself. This necessitated the use of an alternative high-throughput screen. The screen we developed employs fusions of the bioluminescence *luxABCDE* operon to promoters known to be under the regulatory control of *sarA*, thus allowing us to assess activity based on relative levels of bioluminescence as assessed using a 96-well microtiter plate reader. This allowed us to screen 80 compounds, along with positive and negative controls, in a single microtiter plate, thus requiring ~700 plates to complete the analysis of our primary library.

During the course of the current year, we ordered the 31 compounds found to be of greatest interest in our primary screen (see 2012 Progress Report). These were re-screened using our 96-well microtiter plate reporter assay and biofilm assays, but a technical error resulting in a miscalculation of compound concentration precluded meaningful comparisons. At this point, it was necessary to save costs, thereby reserving funds for eventual animal studies, so we limited our focus to the two most promising compounds. We were unable to confirm the utility of one of these compounds in our biofilm assays (data not shown), but we did confirm using a microtiter plate assay that biofilm formation was limited in a concentration-dependent manner in the presence of increasing amounts (5, 10, and 20X) of TimTec compound ST014221. Importantly, this was true in both the *S. aureus* strains UAMS-1, which is methicillin sensitive, and the USA300 isolate LAC, which is methicillin resistant. Controls included each strain assayed in the absence of inhibitor (0) and the isogenic *sarA* mutant.

Importantly with respect to the time frame of our studies, we did encounter another technical difficulty with ST014221 in our standard microtiter plate assay. Specifically, the compound would “stick” to the plastic of the plate, thereby precluding an accurate determination of the relative capacity to form a biofilm. While this caused a delay in our secondary screening efforts, we developed a new assay that allow us to use viable bacterial counts rather than crystal violet staining to assess biofilm formation, and this allowed us to resolve this issue and obtain promising biofilm assay results. More directly, we confirmed a definitive decline in biofilm formation as the concentration of ST014221 increased (see next page). It is also important to note that at these levels (20X being 20ug/ul

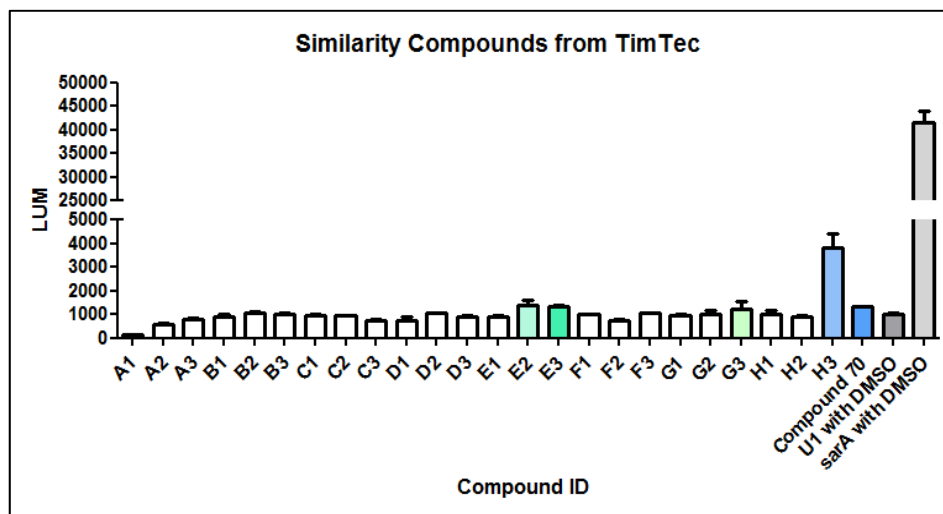


DMSO), the compounds are not toxic. This compound is exactly what we were looking to find in the original compound library of ~30,000. We are continuing to screen the library for comparable compounds, but to date we have not identified any with promise comparable to that of ST014221. Thus, our primary focus over the last year has been on improving the efficacy of TimTec ST014221 *in vitro* in order to ensure that we are in the best position to move forward with *in vivo*.



To this end, we contacted the company that provided the original compounds and obtained a list of chemically similar compounds. We ordered 24 of these compounds and repeated our primary reporter screen. This led to identification of a single compound we have designated H3 (TimTec ST4035075), which yields increased bioluminescence in the parent strain containing the *sspA::luxABCDE* reporter to a statistically significant degree and at a level that is almost 5 times greater than ST014221 (compound 70). Unfortunately, we have been unable to assess the extent to which this translates to inhibition of biofilm formation because compound H3 is not soluble in the medium we use for our biofilm assays. Current efforts are directed toward

both overcoming this issue and exploring medicinal chemistry approaches to optimizing the efficacy of both of these promising compounds. Thus, while neither compound yielded luminescence levels approaching those of the isogenic *sarA* mutant, it is not possible to fully know at this point whether the increased levels observed with these compounds will prove therapeutically relevant, but we are ready to address this issue in our secondary screenings with a specific focus on these compounds. Thus, despite the



unanticipated and unavoidable delays we encountered, we have made significant progress toward our ultimate experimental objectives during the previous funding period.

When taken together, these results put us in a position to move forward with respect to both completing our primary screen and expanding our studies to secondary assays, with the results of the former being incorporated into the latter as the studies progress. This integration of our efforts will ultimately allow us to not only comprehensively identify compounds of potential interest but also rank those compounds by comparison to each other. This will be an important consideration in prioritizing our animal studies. For this reason, we do not anticipate proceeding to animal studies before this milestone is achieved.

FUTURE STUDIES

The compounds we have identified so far as being the most promising, also present some interesting opportunities to because they are related. We have plans to contact chemical experts who specialize in this type of compound chemistry in order to optimize these compounds for *in vivo* studies. The primary emphasis of the Smeltzer laboratory will remain on 1) finishing the TimTec screen, 2) validating promising compounds and ranking them with respect to each other, 3) enhancing the chemical nature of the best compounds, and 4) initiating animal studies with the most promising compounds.

KEY RESEARCH ACCOMPLISHMENTS

1. Identified one compound from the original library that we believe will be to be therapeutically relevant. (ST014221)

2. Identified another related compound from TimTec with greater inhibitory activity than any compounds previously tested. (ST4035075)

REPORTABLE OUTCOMES

The experiments being carried out under the auspices of this project do not involve human subjects and therefore do not have reportable outcomes.

CONCLUSION

By comparison to the original 3 year timetable of this project, the experiments have been delayed owing to the technical issues discussed above. However, these issues have been resolved and we are hopeful that the two compounds we have identified will be therapeutically relevant. By resolving these technical issues, it will also allow us to expand our secondary screen in a manner that will provide further validation of the most promising compounds and allow us to rank them by direct comparison to each other. This will put us in a position to move forward with animal studies focusing on the most promising compounds. Thus, we are confident we will accomplish the objectives of this project, and we are equally confident that the results we ultimately obtain will have a significant impact on the clinical approach and, more importantly, the therapeutic outcome in biofilm-associated infections arising from traumatic injury including those directly associated with military service.

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